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Original Contribution

ANTIPROLIFERATIVE EFFECT OF THE PIPERIDINE NITROXIDE TEMPOL ON NEOPLASTIC AND NONNEOPLASTIC MAMMALIAN CELL LINES

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Abstract—The stable nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) is widely used as a probe in biophysical studies and as an antioxidant in several experimental models. The potential cytotoxic effects of TEMPOL were tested on a panel of human and rodent cell lines, and the nitroxide proved to be significantly more effective in inhibiting the growth of neoplastic than nonneoplastic cell lines after a 96-h exposure. More detailed studies on MCF-7/WT cells indicate that at least 24 h are necessary for TEMPOL to induce irreversible cell damage, which seems to be related to the reactivity of the nitroxyl group. This observation, together with the antagonistic effect of *N*-acetylcysteine, suggests an involvement of free radical-mediated processes. Cell cycle studies indicate a biphasic effect of TEMPOL, with a short-term accumulation of the cells in the G_1 phase and a later increase in G_2/M phase; the pattern of DNA fragmentation observed in TEMPOL-treated cells points to an apoptotic mode of cell death. In conclusion, our data suggest that, while the possible cytotoxic effects of TEMPOL should not be overlooked when using this compound as a biophysical probe or antioxidant, these same properties could be exploited as a novel approach to cancer chemotherapy, especially in tumor cells exhibiting unfavorable characteristics, such as a multidrug-resistant phenotype or loss of hormone receptors. © 1998 Elsevier Science Inc.

Keywords—Cytotoxicity, Tumor cells, Free radicals, Apoptosis, Cell cycle

INTRODUCTION

Low molecular weight nitroxides are free radical compounds widely used in EPR spectroscopy as probes for a number of biophysical and biochemical parameters in biological systems[1–3] and are studied as contrast agents for MRI.[4] In addition, it has recently been demonstrated that piperidine nitroxides exert a cytoprotective action against diverse oxidative insults, including radiation,[5,6] cytotoxic drugs and xenobiotics,[7–9] and postischemic reperfusion injury,[10,11] due to their ability to scavenge oxygen free radicals and to inhibit their generation.[12,13]

In spite of the increasing number of indications for the use of this class of compounds, information concerning their cytotoxicity is sparse. Studies on the effects of several nitroxides on Chinese hamster ovary (CHO) cells have indicated that this cell line is substantially unaffected by a 10-min exposure to most of the compounds tested, at concentrations up to 1 mM.[14] A more recent report on the effects of the piperidine nitroxide 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO) on the growth of three different tumor cell lines suggested that this class of nitroxides can exert a pro-oxidative effect in cells by increasing the intracellular H_2O_2 concentration, even though the same authors conclude that this potentially toxic effect of the nitroxides is overshadowed by its capability to prevent H_2O_2 conversion to the more severely damaging hydroxyl radical.[15]

On the other hand, several six-membered nitroxide free radicals have been found to be mutagenic on the *Salmonella typhimurium* strain TA 104,[16,17] and some of them have been shown to exert bactericidal and bacteriostatic effects on recombination-deficient *E. coli* cells,[18] indicating that these compounds are not completely devoid of toxic effects.

To settle the issue of the potential interference of

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nitroxide free radicals with cell viability and proliferation, in the present study we have examined the effects of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), which is a widely used antioxidant piperidine nitroxide,[6,9] on a panel of cultured cell lines, derived from different organs and tumors of human and rodent origin. Our results indicate that (a) upon exposure times longer than 24 h TEMPOL exerts a significant inhibitory action on cell growth, possibly by triggering an apoptotic mechanism; (b) this effect appears to be more potent on neoplastic cells than on isotypic nontumor cells; (c) the effect of TEMPOL is not impaired in cells displaying a multidrug resistant phenotype or (in the case of breast cancer cells) lacking estrogen receptors; (d) growth inhibition is associated with dose- and time-dependent alterations of cell cycle progression; (e) the antiproliferative action of TEMPOL appears to depend on the presence of an unpaired electron in the nitroxide molecule and on the reduction rate of the compound, and is significantly reduced by combination with the free radical scavenger *N*-acetylcysteine (NAC).

MATERIALS AND METHODS

Chemicals

4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), *N*-acetyl-L-cysteine (NAC), 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (CTPY), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B (SRB), and all other reagents were purchased from Sigma-Aldrich s.r.l. (Milan, Italy). TEMPOL hydroxylamine (TEMPOL-H), generously provided by Dr. M. K. Cherukuri (Radiation Oncology Branch, N.C.I., N.I.H., Bethesda, MD), was prepared by hydrogenation in the presence of a platinum catalyst.[19]

Cell lines and culture conditions

The following cell lines and culture media were used: HBL-100 (human breast epithelium), maintained in McCoy medium, supplemented with 10% fetal bovine serum (FBS); MCF-7/WT (human breast adenocarcinoma, estrogen receptor positive), MCF-7/ADR (MDR variant of MCF-7/WT selected for resistance to doxorubicin), MDA-MB-231 (human breast adenocarcinoma, estrogen receptor negative), and NIH: OVCAR-3 (human ovarian adenocarcinoma), maintained in RPMI-1640 medium supplemented with 10% FBS; CHO-K1 (Chinese hamster ovary), LoVo/WT (human colon adenocarcinoma), LoVo/DX (MDR variant of LoVo/WT selected for resistance to doxorubicin) and BRL-3A (rat liver), maintained in Ham's F12 medium supplemented with 10% FBS; MH1-C1 (rat hepatoma), maintained in Ham's F10 medium supplemented with 5% FBS and 15% horse serum; HCT 116 (human colon adenocarcinoma) maintained in DMEM supplemented with 10% FBS.

All the cell lines were maintained under standard culture conditions at 37°C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assays

The effects of TEMPOL, CTPY, and TEMPOL-H on cell proliferation were determined by the MTT assay, as described by Alley et al.;[20] to avoid auto oxidation, TEMPOL-H was prepared as a stock solution containing 50 μM diethylenetriaminepenta-acetic acid (DETAPAC). Briefly, cells were seeded onto 96-well plates and allowed to grow for 24 h prior to treatment with different concentrations of the nitroxide. After 96 h, MTT (5 mM in PBS) was added to the cells for 3 h at 37°C. Formazan crystals, formed by mithocondrial reduction of MTT, were solubilized in DMSO and the absorbance was read at 570 nm. Preliminary experiments were performed to verify the correlation between MTT reduction and total cell counts. Because MTT interacts directly with sulfhydryl compounds, the effect of NAC on TEMPOL cytotoxicity could not be evaluated by this assay; therefore, a colorimetric assay based on protein staining by sulforhodamine B (SRB) was used instead.[21] Cells were seeded and treated as described for the MTT assay. After a 96-h treatment, cells were fixed with trichloroacetic acid (final concentration 10%), stained with 0.4% SRB and washed with 1% acetic acid. The incorporated dye was solubilized in 10 mM Tris (pH 10–10.5), and absorbance was read at 550 nm. IC_{50} values were determined by using the median-effect equation.[22]

To assess the time course for the development of TEMPOL effects on cell growth, MCF-7/WT cells were exposed to TEMPOL according to different time schedules: (a) 2-h exposure to TEMPOL, MTT assay performed 94 h after drug removal; (b) 24-h exposure to TEMPOL, MTT assay performed immediately thereafter; (c) 24-h exposure to TEMPOL, MTT assay performed 72 h after drug removal; (d) 96-h exposure to TEMPOL, MTT assay performed immediately thereafter.

The 96-h end point was chosen because it yielded optimal values for the optical density in control wells. Phase contrast micrographs (250×) of MCF-7/WT control cells and of cells treated with TEMPOL according to schedules (b) and (d), were taken on Agfa Ortho 135 film.

Table 1. Antiproliferative Effect of TEMPOL on Different Human and Rodent Neoplastic and Nonneoplastic Cell Lines

Cell Line	Tumorigenic Potential	MDR Phenotype	IC_{50} ± SE (mM)
Breast			
HBL-100	−	−	0.944 ± 0.082
MCF-7/WT	+	−	0.208 ± 0.023*
MCF-7/ADRR	+	+	0.410 ± 0.048*
MDA-MB-231	+	−	0.464 ± 0.063*
Colon			
LoVo/WT	+	−	0.499 ± 0.039
LoVo/DX	+	+	0.303 ± 0.059
HCT 116	+	−	0.380 ± 0.060
Liver			
BRL-3A	−	−	1.073 ± 0.070
MH1-C1	+	−	0.773 ± 0.038†
Ovary			
CHO-K1	−	−	0.891 ± 0.227
NIH: OVCAR-3	+	−	0.222 ± 0.020‡

Mean ± SE of four to six experiments.
Statistically significant differences were assessed by the analysis of variance, followed by Duncan's test for multiple comparisons.
* $p < 0.05$ vs. HBL-100.
† $p < 0.05$ vs. BRL-3A.
‡ $p < 0.05$ vs. CHO-K1.

under an inverted microscope equipped with a MPS 51S camera.

EPR studies

The stability of a 0.1 mM solution of TEMPOL in complete culture medium (RPMI 1640 with 10% FBS) was assessed up to 96 h, corresponding to the longest exposure time in the study. TEMPOL stability in the intracellular environment was also evaluated. Cells were seeded onto 75 cm^2 flasks and allowed to attach for 24 h prior to treatment with 0.1 mM TEMPOL. After 24 h, the drug-containing medium was removed and cells were trypsinized, washed in PBS, and resuspended at 1×10^6 cells/ml; cell samples were drawn into gas permeable Teflon capillaries (inner diameter 0.8 mm, wall thickness 0.38 mm; Zeus Industries, Raritan, NJ) and analyzed by EPR to determine the amount of TEMPOL at the end of the incubation. The time course of the decay of the EPR signal of TEMPOL in MCF-7/WT cells was followed by processing cells from separate flasks at different time points after replacing the TEMPOL-containing medium with fresh drug-free medium.

EPR spectra of TEMPOL-containing medium and of cell suspensions were obtained at room temperature under the following conditions: microwave power 5 mW, gain 5×10^2, time constant 0.5 s, modulation amplitude 1 G.

Cell cycle analysis

MCF-7/WT cells were seeded onto 75 cm^2 flasks and allowed to attach for 24 h prior to treatment with different concentrations of TEMPOL (0.4, 0.8, and 1.2 mM for 24-h treatments and 0.1, 0.2, and 0.4 mM for 96-h treatments); untreated cells, grown for the same periods of time, were used as controls. At the end of the appointed treatment periods cells were harvested by trypsinization, washed in ice-cold PBS, fixed in 70% ethanol, and stored at −20°C. Just prior to analysis by FACS, cells were washed and resuspended at 1×10^6 cells/ml in PBS. Cellular DNA was stained with 30 μg/ml propidium iodide in PBS in the presence of RNAse A at 37°C for 30 min. Cell cycle studies were performed using a FACScan flow cytometer (Becton Dickinson) and the data were analyzed by Lysis II version 1.1. Results are reported as the number of cells vs. their DNA content, as indicated by the intensity of fluorescence.

DNA fragmentation

Internucleosomal DNA fragmentation was analyzed by conventional agarose gel electrophoresis. MCF-7/WT and HBL-100 cells were treated for 24 h with different concentrations of TEMPOL. At the end of this period, the cells (5×10^6/sample) were lysed by incubation in lysis buffer (10 mM Tris-HCl, pH 8; 150 mM NaCl, 20 mM EDTA, and 0.5% SDS) and the resulting lysates were incubated overnight with 0.5 mg/ml proteinase K at 50°C. After incubation with

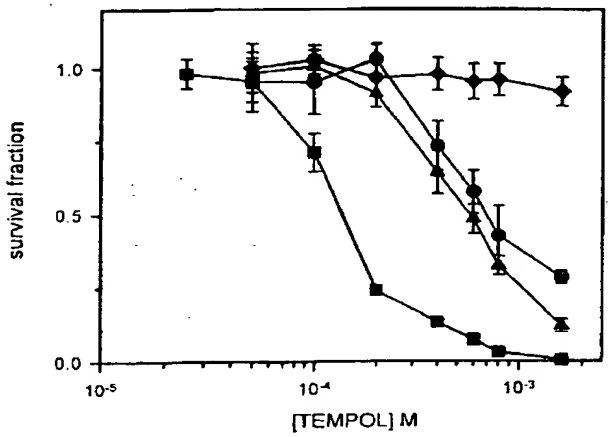


Fig. 1. Antiproliferative effects of TEMPOL on MCF-7/WT cells after different exposure times: ◆ 2-h TEMPOL + 94 h in TEMPOL-free medium before the MTT assay; ▲ 24-h TEMPOL and MTT assay immediately thereafter; ● 24-h TEMPOL + 72 h in TEMPOL-free medium before the MTT assay; ■ 96-h TEMPOL and MTT assay immediately thereafter. Each curve is representative of three to four independent experiments.

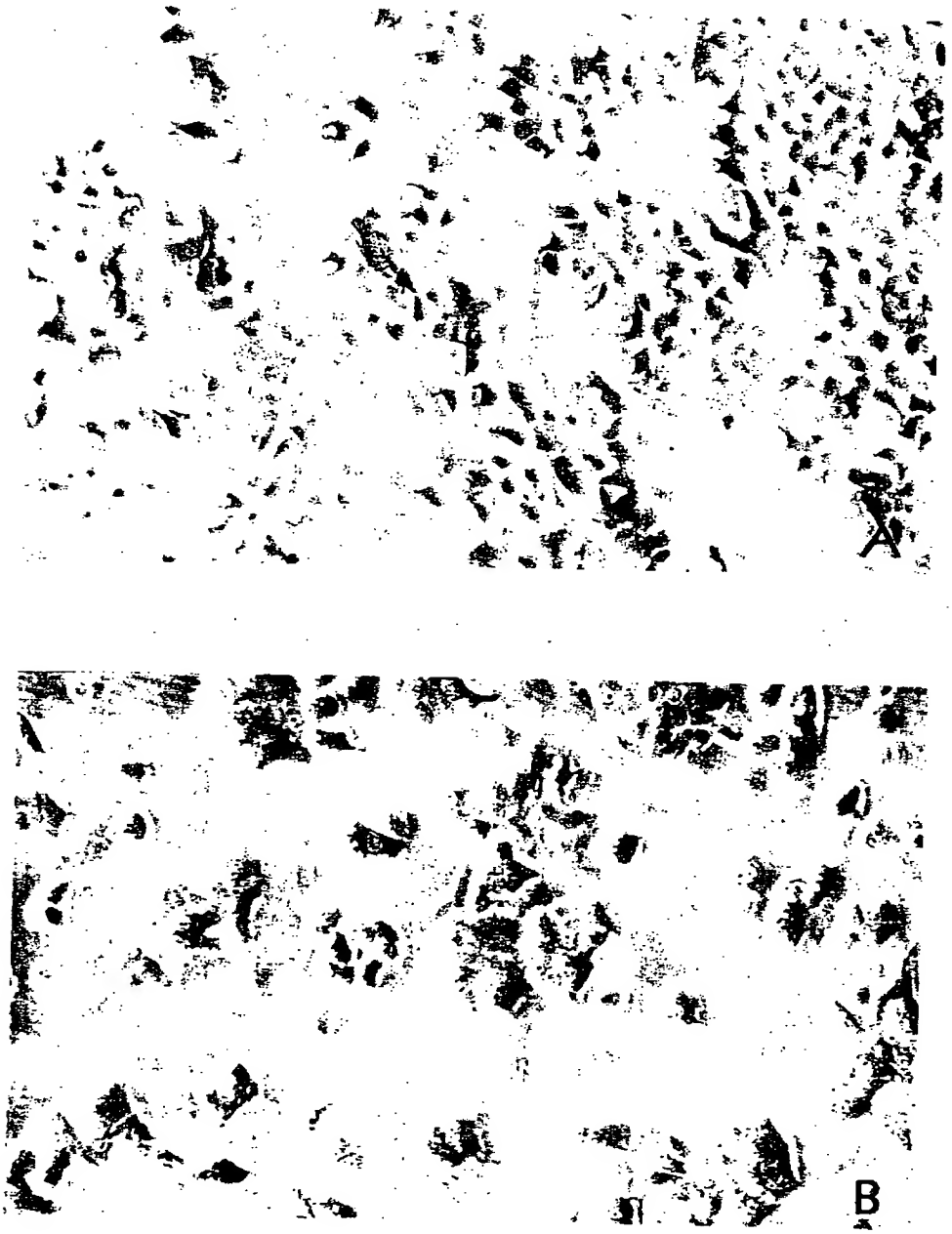


Fig. 2. Effect of TEMPOL on MCF-7/WT cells. (A) control, 24 h; (B) 1.2 mM TEMPOL for 24 h; (C) control, 96 h; (D) 0.4 mM TEMPOL for 96 h (200× magnification).

$NaCH_3COOH$ 3M (1/10 v/v) and ethanol (2/1 v/v) for 30 min on ice, the DNA was recovered by centrifugation at 14,500 × *g* for 30 min at 4°C and the resulting pellet was dissolved in TE buffer (Tris 10 mM, EDTA 1 mM, pH 7.5) containing 0.1 mg/ml ribonuclease A for 1 h at 37°C. The samples thus obtained were then resolved by electrophoresis on 2.0% agarose gels at 10 V/cm for 120 min and DNA was visualized by exposure of the gel to UV light after incubation with ethidium bromide (0.5 μg/ml) for 1 h.

Statistical analysis

Data from cytotoxicity studies were analyzed by a one-way analysis of variance; multiple comparison among IC_{50} values were evaluated by means of Duncan's

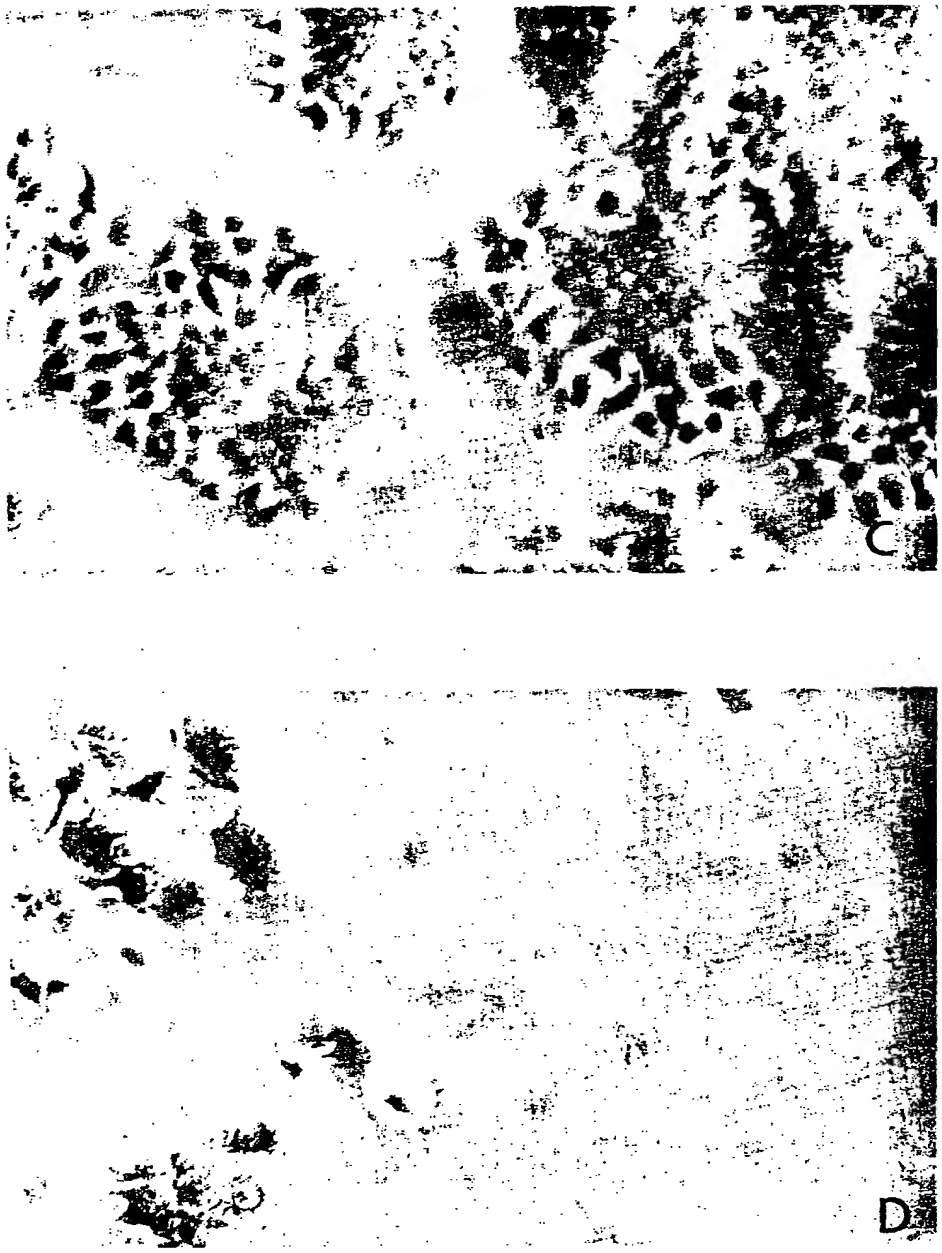


Fig. 2. *Continued.*

multiple range test ($p < .05$). The type of interaction between TEMPOL and NAC was evaluated by the method described by Drewinko.[23]

RESULTS

Cytotoxicity tests

Table 1 shows the IC_{50} values obtained on a panel of human and rodent cell lines for a 96-h exposure to TEMPOL. Whenever possible, the MTT test was used to assess the effects of TEMPOL on cell growth, based on preliminary experiments showing that MTT reduction was closely correlated to total cell counts and protein staining with SRB, following exposure to the nitroxide (data not shown). Because NAC interacts directly with MTT, the SRB assay was used whenever NAC was included in the experimental protocol.

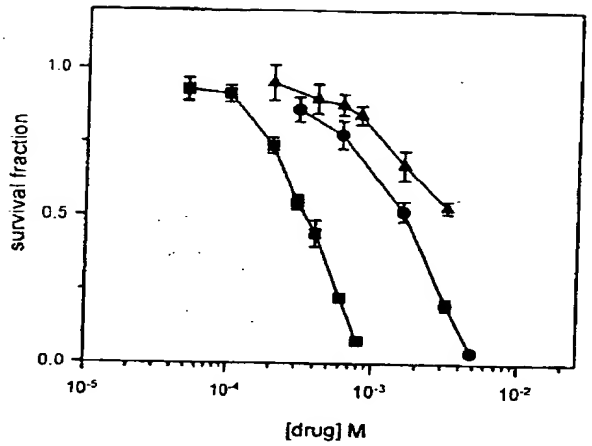


Fig. 3. Antiproliferative effects of TEMPOL (■), TEMPOL hydroxylamine (▲) and 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (●) on MCF-7/WT cells upon 96-h exposure. Each curve is representative of three to four independent experiments.

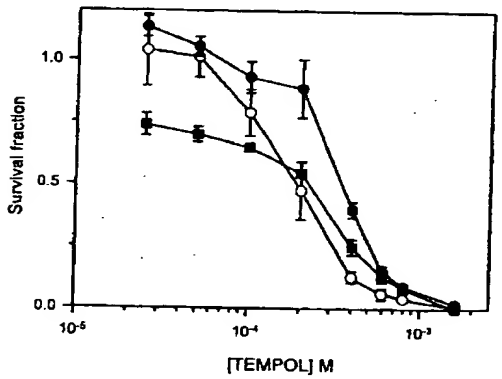
The following observations can be made: (a) whenever a comparison is possible between isotypic tumor and nontumor cell lines (breast, liver, and ovary), TEMPOL is significantly more cytotoxic against the former vs. the latter. (b) No significant differences in TEMPOL cytotoxicity can be detected between cell lines exhibiting a multidrug-resistant phenotype (MCF-7/ADR and LoVo/DX) and the corresponding parental cell lines (MCF-7/WT and LoVo/WT, respectively). (c) No significant differences in TEMPOL cytotoxicity can be detected between breast cancer cell lines with (MCF-7/WT) or without (MCF-7/ADR, MDA-MB-231) estrogen receptors.

All subsequent studies were performed on MCF-7/WT cells, which are the most sensitive to the effects of TEMPOL of all the cell lines tested.

Figure 1 shows the time course of the antiproliferative effects of TEMPOL on MCF-7/WT cells. A 2-h exposure to the nitroxide, followed by its removal from the culture medium, does not affect cell proliferation, as assessed by the MTT assay 96 h after the beginning of drug treatment, suggesting that no irreversible cell damage is induced during this period of time. After 24-h exposure to the nitroxide, a dose-dependent inhibition of cell growth begins to develop; no significant differences are apparent between the results of a cytotoxicity assay performed immediately at the end of 24 h (IC_{50} 0.764 ± 0.097 mM, mean ± SD) and the same test performed after the cells have been allowed to recover for 72 h in TEMPOL-free culture medium (IC_{50} 0.604 ± 0.208 mM, mean ± SD), indicating that 24 h are enough to induce irreversible damage to the cells. The contrast phase micrographs shown in Fig. 2a and b support this finding, showing significant morphologic alterations in cells exposed to 1.2 mM TEMPOL for 24 h compared to control cells grown in drug-free medium for the same period of time. Continuous exposure to the nitroxide for 96 h results in a more potent inhibition of cell growth (IC_{50} 0.208 ± 0.06 mM, mean ± SD). An increased severity of the morphologic lesions is observed when cells are exposed to 0.4 mM TEMPOL for 96 h compared to appropriate control cells (Fig. 2c and d).

The experiments reported in Fig. 3 were performed in MCF-7/WT cells to assess the importance of the nitroxyl group in the cytotoxicity of TEMPOL. The dose–response curve obtained for a 96-h exposure to TEMPOL is compared with those obtained with TEMPOL-H (its chief metabolite, in which the nitroxyl group is reduced to hydroxylamine[24]), and CPTY, another nitroxyl compound that exhibits a lower reduction rate than TEMPOL and therefore is less reactive at physiological pH.[19] Both TEMPOL-H and CPTY are significantly less potent in inhibiting MCF-7/WT cell growth than TEMPOL (IC_{50} 2.33 ± 1.12 mM and 1.05 ± 0.15 mM, respectively; mean ± SD), indicating that a highly reactive nitroxyl group is essential for the cytotoxic activity of the compound.

When different concentrations of *N*-acetylcysteine (NAC) are combined with TEMPOL for the in vitro treatment of MCF-7/WT cells (Fig. 4), the cytotoxic



[NAC] mM	% growth inhibition	TEMPOL IC_{50} (mM) in the presence of NAC (Mean ± SD)
0	–	0.208 ± 0.023
5.0	0.0	0.329 ± 0.069
10.0	22.2	0.273 ± 0.077

Fig. 4. Effect of *N*-acetylcysteine (NAC) on the growth inhibitory action of TEMPOL on MCF-7/WT cells upon 96-h exposure (mean ± SD of four to six replications). ■ TEMPOL; ○ TEMPOL + 5.0 mM NAC; ● TEMPOL + 10 mM NAC. Each curve is representative of three to four independent experiments.

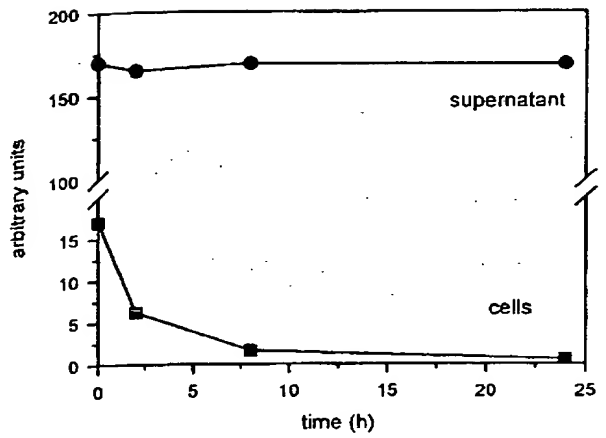


Fig. 5. Stability of the TEMPOL molecule in culture medium (●) and in cells (■). RPMI 1640 medium (supplemented with 10% FBS) containing 0.1 mM TEMPOL was incubated for 96 h under standard cell culture conditions; EPR spectra were recorded at different time points. MCF-7/WT cells were exposed for 24 h to 0.1 mM TEMPOL under standard culture conditions. The nitroxide was subsequently removed from the extracellular medium and EPR spectra of the cell suspension were recorded at different time points after drug removal. All EPR spectra were recorded at room temperature under the following conditions: microwave power 5 mW, gain 5×10^2, time constant 0.5 s, modulation amplitude 1.

action of TEMPOL, as assessed by the sulforhodamine B assay, is inhibited: a significant increase in the IC_{50} value of TEMPOL (0.329 ± 0.069 mM, mean ± SD vs. 0.208 ± 0.06 mM in the absence of NAC) was observed in the presence of 5 mM NAC, which per se is devoid of appreciable cytotoxic effects, whereas an IC_{50} value of 0.273 ± 0.077 mM (mean ± SD) was obtained in the presence of 10 mM NAC, which induces ≈25% growth inhibition when given alone. Preliminary studies had shown that, at the concentrations used for these experiments, NAC does not affect the intensity of the TEMPOL EPR signal in a cell-free system (data not shown), indicating that the two compounds do not interact directly and that any interactions between the two occur intracellularly. The statistical analysis of the combined effects of TEMPOL and NAC 10 mM by the test described by Drewinko[23] indicates a significant antagonism of TEMPOL action by this concentration of NAC.

EPR studies

The stability of the TEMPOL molecule for 96 h (corresponding to the longest exposure time used in our experiments), was assessed both in tissue culture medium in the presence of serum components and in the intracellular environment, by measuring the intensity of TEMPOL EPR signal. Figure 5 shows that the signal corresponding to 0.1 mM TEMPOL in RPMI 1640 tissue culture medium containing 10% fetal bovine serum is essentially unmodified during the first 24 h and that 85% of the initial signal is still detectable after 96 h. The time course of TEMPOL signal decay in MCF-7/WT cells preincubated with 0.1 mM TEMPOL and subsequently maintained in TEMPOL-free medium shows that the intracellular concentration of the nitroxide rapidly decreases and is no longer measurable after 24 h.

Cell cycle studies

Flow cytometric studies of the DNA content of TEMPOL-treated MCF-7/WT cells and their untreated counterparts were performed to assess the effect of TEMPOL on the cell cycle progression. Figure 6 shows the flow cytometric analysis of the cell cycle distribution of MCF-7/WT cells exposed to different concentrations of TEMPOL for 24 and 96 h. At 24 h control cells show a DNA content distribution representative of an asynchronous, exponentially growing cell population; exposure to TEMPOL induces a dose-dependent accumulation of cells in the G_1 phase. After 96 h the DNA content distribution of control cells is dramatically modified, with a significant increase in G_1 cells as the population approaches the plateau phase; at high nitroxide concentrations a fraction of cells with a hypodiploid DNA content can be detected. Upon long exposure times, TEMPOL no longer causes cells to accumulate in G_1, but rather depletes this cell subpopulation, with a corresponding increase in G_2/M cells.

DNA fragmentation

Figure 7 shows the effect of TEMPOL on DNA fragmentation in the human breast adenocarcinoma cell line MCF-7/WT (lanes 1–3) and in the nonneoplastic human breast epithelial cell line HBL-100 (lanes 4–6) after 24-h exposure TEMPOL concentrations up to 2.5 mM fail to induce any detectable DNA degradation in HBL-100 cells (lanes 5 and 6), whereas upon 24-h exposure to 2.5 mM TEMPOL MCF-7/WT cells yield a pattern of internucleosomal DNA fragmentation (lane 3), which is typical of apoptotic cell death.

DISCUSSION

The increasing use of low molecular weight nitroxides as probes in EPR and NMR imaging[1–4] and as protective agents in a number of free radical-mediated pathologies[5–11] is based on the assumption that these compounds are devoid of toxic effects per se, and therefore unable to induce significant cellular damage. Studies supporting this assumption have indicated that short-

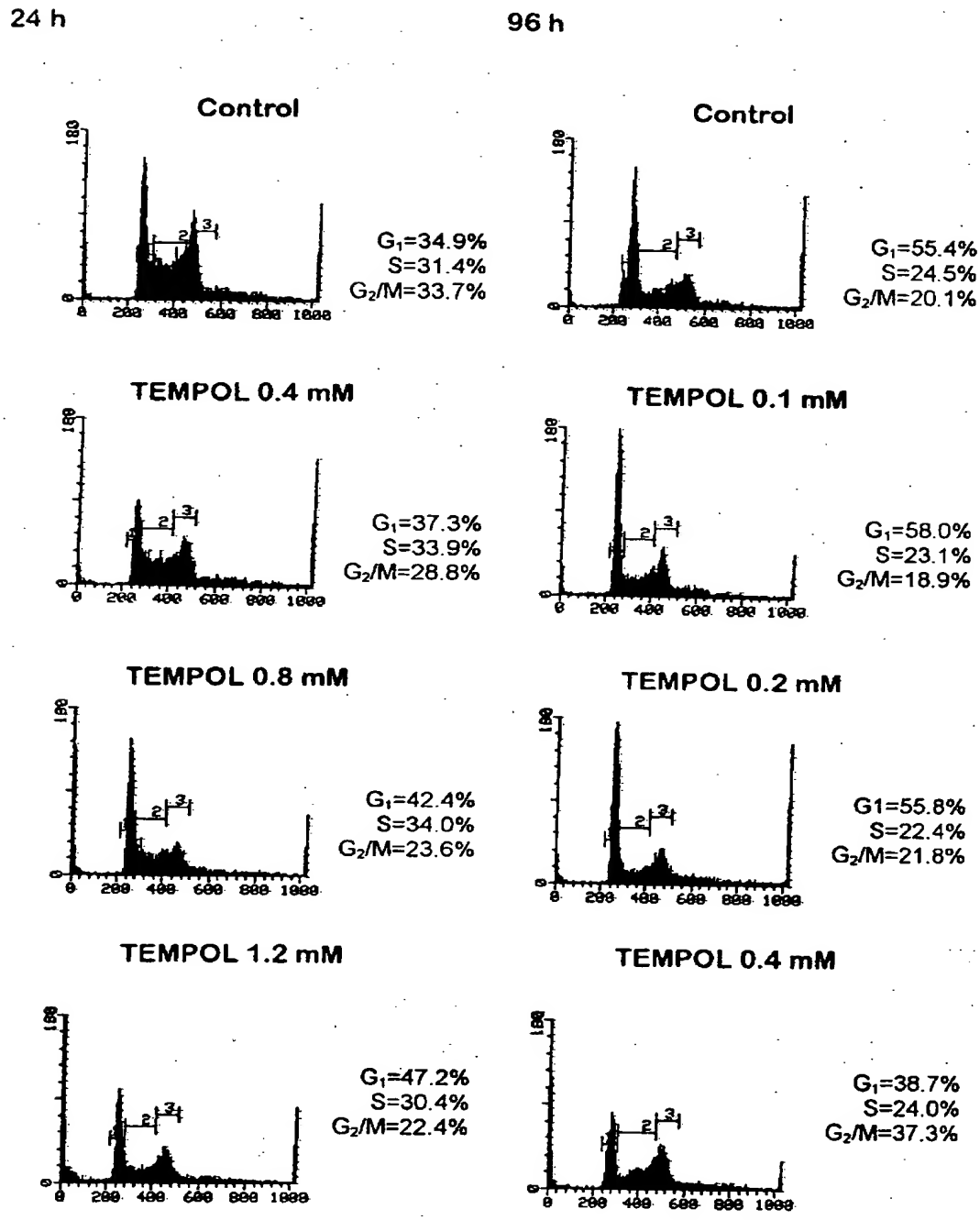


Fig. 6. Flow cytometric analysis of the cell cycle distribution of MCF-7/WT cells exposed to different concentrations of TEMPOL for 24 and 96 h. Histograms and percent values of cell distribution in the different phases of the cell cycle refer to a single experiment, which is representative of three replicate experiments with similar results.

term exposure (10 min) of CHO cells to a variety of low molecular weight nitroxides does not affect cell viability,[14] and that no significant cytotoxicity can be observed when tumor cells are treated with the piperidine nitroxide TEMPO, in spite of the production of substantial amounts of hydrogen peroxide.[15] However, other studies testify to the mutagenic effects of TEMPOL;[16,17] a very recent report indicates that nitroxide free radicals have bactericidal/bacteriostatic properties against some bacterial strains, depending on their repair capability,[18]

Fig. 7. DNA fragmentation assay in MCF-7/WT and HBL-100 cells exposed to TEMPOL for 24 h. Lanes 1–3: MCF-7/WT cells (controls, TEMPOL 1.0 mM, and 2.5 mM, respectively); lanes 4–6: HBL-100 cells (controls, TEMPOL 1.0 mM, and 2.5 mM, respectively).

suggesting that more thorough investigations on the effects of TEMPOL on cell survival and proliferation might be in order.

The apparent paradoxical effect of TEMPOL, which seems to act as an antioxidant cytoprotective agent in some experimental models,[5–11] while exhibiting a cytotoxic action under different settings, prompted us to perform a thorough investigation of the effects of TEMPOL on cell survival and proliferation, using a panel of cell lines derived from different organs and tumors of human and rodent origin. Our findings point to a marked cytotoxic effect for TEMPOL, but not all the cell lines tested are equally affected by the treatment. The most striking differences can be observed between neoplastic and nonneoplastic cell lines from breast, liver, and ovary, with a significantly more potent effect against neoplastic cells. This observation suggests the attractive possibility that TEMPOL may exhibit some degree of selectivity as a cytotoxic agent. The mechanisms underlying this comparatively selective effect are unclear as yet. On the one hand, evidence has been presented that tumor cells are under a latent oxidative stress, which is accompanied by deficit in the activity of detoxifying enzyme systems,[25] and which, at least in some cases, has been shown to be essential for the maintenance of a transformed phenotype.[26] If such were the case, the antiproliferative effect of TEMPOL could be attributed to a relief of the intracellular oxidative stress by virtue of its antioxidant properties. Alternatively, it could be envisioned that TEMPOL, when present in excess to the intracellular free radical level, might itself act as a free radical, and attack cellular components essential to cell survival, directly or by generating oxygen-derived reactive species.[15] Either way, the growth inhibitory effect of TEMPOL seems to depend on free radical-mediated processes; this hypothesis is supported by the results obtained in our experiments combining TEMPOL and NAC, which acts as a precursor for GSH and as a free radical scavenger in its own right. Preliminary EPR studies have demonstrated that TEMPOL signal is not affected by NAC in a cell-free experimental system (data not shown), thus ruling out a direct interaction between the two compounds. Therefore, the NAC-mediated inhibition of TEMPOL effects can be hypothesized to occur only in the presence of cellular components.

The importance of free radical-mediated mechanisms to obtain a significant inhibition of cell growth is emphasized by the results shown in Fig. 3, suggesting that TEMPOL owes its antiproliferative effect not only to the presence, but also to the reactivity of its nitroxyl group. Accordingly, TEMPOL-H, which is the chief reduced metabolite of TEMPOL,[24] was shown to be almost totally devoid of cytotoxicity against MCF-7/WT cells, with a negligible residual activity probably due to reoxidation of the hydroxylamine to nitroxyl group.[27] Likewise, CTPY, which is a nitroxide with a higher midpoint potential than TEMPOL and a decreased reduction rate at physiological pH,[19] proved to be significantly less effective than TEMPOL in inhibiting MCF-7/WT cell growth. The possible role played by the reactive nitroxyl group in triggering free radical-mediated cell damage suggests that cells better equipped to detoxify free radicals and their reaction products (e.g., lipoperoxides) are less responsive to TEMPOL cytotoxicity. The observation that both the tumor and nontumor cell lines derived from rat liver, exhibiting high levels of detoxifying enzymes, are comparatively more resistant to the effects of TEMPOL than the cell lines derived from other organs, provides further support to this hypothesis. Other factors accounting for the effects of TEMPOL in individual cell lines include cell pharmacokinetics (uptake and metabolism) of the compound, DNA repair capability of the cell line tested as well as its ability to set up a program of apoptotic cell death, based on the expression of such genes as *bcl-2*, *bax*, *c-myc*, and *p53*. None of these issues has been specifically addressed in the present study, which was only meant as a preliminary characterization of the effects of TEMPOL on mammalian cell growth. However, indirect evidence seems to rule out a major role for mismatch repair enzymes, because the response of the two colon cell lines tested, LoVo and HCT 116, which harbor defects in mismatch repair components (hMSH2 and hMLH1 respectively),[28,29] does not signif-

icantly differ from the response observed in the other tumor cell lines in the panel.

A further issue of considerable interest is the irreversible character of the cell damage induced by TEMPOL. This effect was studied in greater detail in MCF-7/WT cells, which were especially responsive to the nitroxide. Experiments based on different treatment schedules show that the cytotoxic effect of TEMPOL is time-dependent, and that 24 h are required for the development of cell damage, as assessed by morphological alterations and loss of proliferative capacity. No appreciable recovery of proliferative capacity is observed upon replacement of TEMPOL with drug-free medium after 24-h exposure; on the contrary, a slight increase in cytotoxicity can be detected, indicating that drug effects persist even after removal of the compound from the extracellular space. This observation can be accounted for by different explanations, the most obvious being that part of the TEMPOL incorporated by the cells persists in the intracellular compartment throughout the experiment, even after nitroxide removal from the extracellular space. However, our EPR studies rule out this possibility, as the TEMPOL signal was undetectable in cells shortly after its removal from the extracellular medium. The alternative explanation, based on intracellular conversion of TEMPOL to a diamagnetic, EPR-silent compound, which nevertheless might retain its antiproliferative effect, is ruled out by the results of the experiments reported above, showing that TEMPOL-H is devoid of cytotoxic properties. Therefore, the most likely explanation seems to be that the antiproliferative activity of TEMPOL depends on the presence of a reactive nitroxyl group, which induces irreversible damage.

Cell cycle studies were also performed in MCF-7/WT cells to evaluate whether TEMPOL is able to affect cell progression through any particular phase of the division cycle. Our results indicate a biphasic effect of TEMPOL, with a short-term dose-dependent accumulation of the cells in the G_1 phase of the cell cycle and a decrease of the percentage of cells in this same phase upon longer exposure times, with a concomitant increase in G_2/M cells. This suggests the presence of a TEMPOL-induced damage assessment mechanism, which is associated both with cell cycle progression regulatory mechanisms and with the triggering of cell death. If the damage is recognized as repairable, then cells slow down their progression through the cell cycle and attempt to repair the damage. In contrast, when the damage is extensive and impossible to repair, which seems to be the case after long-term exposure or after 24-h exposure to high concentrations of TEMPOL, cell death is triggered and cells in the G_1 phase of the cell cycle are the first to be killed. As to the mode of the cell death induced by TEMPOL, the presence of a fraction of cells with hypodiploid DNA content after 24-h exposure to high concentrations of the nitroxide suggests the triggering of an apoptotic mechanism may be. This is supported by the results of the DNA fragmentation studies, showing internucleosomal DNA fragmentation in MCF-7/WT, but not in HBL-100, cells exposed to TEMPOL for 24 h. Further investigations are in progress to establish whether this is due to direct DNA damage by TEMPOL, or to other effects of the nitroxide that ultimately result in DNA degradation.

Irrespective of the specific mechanism by which the final outcome is achieved, it can be concluded that TEMPOL is not devoid of effects on mammalian cell growth, as originally proposed by some authors. This possibility should be given thorough consideration, both as a hindrance to the use of nitroxides as probes or antioxidants and as a further indication for the therapeutic use of these compounds, which is made particularly attractive by their comparative selectivity for tumor cells and irreversible character of their cytotoxic effect. In addition, our findings indicate that TEMPOL does not discriminate between tumor cells exhibiting a multidrug-resistant phenotype and the corresponding parental cell lines, and no significant difference has been observed between estrogen receptor-positive and -negative breast cancer cells. As multidrug resistance and loss of hormone receptors in tumor cells are often related to an unfavorable prognosis, the efficacy displayed by TEMPOL against cell lines with these characteristics is a further potential benefit of the therapeutic use of this class of compounds.

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